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14. ABSTRACT

The Neurofibromatosis type 1 (NF1) gene encodes a large tumor suppressor protein, neurofibromin, which is a Ras GTPase-activating protein (RasGAP) activity. Although the NF1 gene was identified over a decade ago, the biological roles of neurofibromin in cellular processes remain unclear. Therefore it is crucial for therapy and developing new drugs for NF1 patients to elucidate how the RasGAP activity of neurofibromin is controlled. To achieve this goal, it is also important to identify regulatory elements for neurofibromin. We are investigating the molecular mechanisms by which the Ras GAP activity of the yeast neurofibromin homologs Ira1/2 is regulated as a model to understand human NF1. We have found that the kelch Gb subunit mimics Gpb1/2 interact with Ira1/2 and control the Ras GAP activity of Ira1/2. Here, we found that the Gpb1/2 proteins are localized to the cell membrane in a Gpa2 dependent manner and function at the cell membrane. Gpb1/2 bind to the C-terminus of Ira1/2 and stabilize the Ira1/2 proteins. Moreover we also identified a Gpb1/2 binding domain near the C-terminus of Ira1/2 (GBD) that is significantly conserved in neurofibromin homologs, including a human counterpart. Therefore, similar regulatory mechanisms might be conserved in evolution.

15. SUBJECT TERMS
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Introduction

Neurofibromatosis type 1 (NF1) is one of the most common genetic disorders in humans and the Ras GTPase-activating protein (RasGAP) neurofibromin is intimately associated with NF1. It is therefore critical to elucidate the molecular mechanisms by which the RasGAP activity of neurofibromin are regulated, as well as the biological roles of neurofibromin, which are as yet incompletely understood. We employ the budding yeast *Saccharomyces cerevisiae* and model fungus *Cryptococcus neoformans* as models to understand how the GAP activity of the yeast neurofibromin homologs, Ira1 and Ira2, is governed. The biochemical and biological roles of the yeast homologs are evolutionarily well conserved.

Recently, we identified the kelch Gβ mimic proteins Gpb1 and Gpb2 (also known as Krh1 and Krh2), which are structurally and functionally related to Gβ subunits yet share no primary sequence identity with known Gβ subunits (Harashima and Heitman, 2002, 2005). Gpb1/2 bind to Ira1/2 in vivo and regulate cAMP signaling by inhibiting the Gα subunit Gpa2 and concomitantly activating the Ira1/2 RasGAPs. In the approved Statement Of Work (See appendices), we proposed to elucidate the roles of the Gβ mimic kelch proteins Gpb1/2 in regulating the yeast neurofibromin homologs Ira1/2 for the first years of this project. We have found that Gpb1/2 are localized to the cell membrane in a Gpa2-dependent manner (Harashima and Heitman, 2005, *Molecular Biology of the Cell*) and function at the cell membrane where Gpb1/2 bind to the C-terminus of Ira1/2 and stabilize the Ira1/2 proteins. These studies were published in *Molecular Cell* (Harashima et al, 2006). These findings set the stage for studies to examine NF1 and possible mammalian kelch protein homologs of Gpb1/2, and extension of these studies to

homologs in other fungi, and to other components of the Gpb1/2-protein kinase A signaling cascade. We summarize here our findings, and focus on progress since the last report and plans for the time remaining.

Body

In our studies, we have identified a novel type of G protein beta subunit mimic that physically interacts with the yeast neurofibromin homologs Ira1 and Ira2, governing their stability and thereby signaling via the Ras-cAMP-protein kinase A signaling pathway. These studies provide considerable new insight into the ways in which novel signaling components are integrated into signaling circuits governing cell growth and cell fate transitions during development, and have resulted in a series of three publications (Harashima and Heitman, 2002; Harashima and Heitman, 2005; Harashima et al, 2006).

To determine whether the functions of the kelch proteins are evolutionarily conserved, we have extended our studies to another genetically tractable fungal model system, *Cryptococcus neoformans*, and identified two kelch repeat homologs that are involved in mating (Kem1 and Kem2). To find kelch-repeat proteins involved in G protein signaling, *Cryptococcus* homologues of Gpb1/2, which interacts with and negatively regulates the G protein alpha subunit, Gpa2, in *S. cerevisiae*, were searched by BLAST (tblastn) in *Cryptococcus* genome database of serotype A (Duke University Medical Center (center for genome technology, http://cneo.genetics.duke.edu/) or the Whitehead Institute Center for Genome Research, http://www-genome.wi.mit.edu/annotation/fungi/cryptococcus_neoformans/index.html) or serotype D (TIGR (http://www.tigr.org/tdb/e2k1/cna1/) or Stanford Genome Technology Center (SGTC, http://www-sequence.stanford.edu/group/C.neoformans/) database). However, Gpb1 and Gbp2 homologues were not found in serotype A or D *Cryptococcus neoformans* genome. Therefore, kelch-repeat proteins involved in mating of other fungi were investigated. In fission yeast *Schizosaccharomyces pombe*, a kelch-repeat protein,

Ral2 (Ras-like), is involved in cell morphology, conjugation and sporulation upstream of Ras1. A BLAST search of the *Cryptococcus* genome database showed that both serotype A and D *Cryptococcus neoformans* contain Ral2 homologues. Interestingly, *S. cerevisiae* seems not appear to have any Ral2 homologues. Instead, the kelch-repeat containing amino terminal half of Kel1 (Kelch-repeat protein 1) is homologous to *S. pombe* or *C. neoformans* Ral2. *S. cerevisiae* Kel1 is involved in cell morphology and mating. Based on BLAST searches, *C. neoformans* has genes encoding hypothetical proteins homologous to the kelch-repeat containing amino terminus of Kel1. Here we name the genes encoding these kelch repeat containing proteins *KEM1* and *KEM2* (Kelch repeat proteins involved in mating), respectively. Therefore, Kem1 and Kem2 are homologuous to *S. pombe* Ral2 and the amino terminal half of *S. cerevisiae* Kel1.

We disrupted all three genes (*KEM1*(*RAL2*), *KEM2*(*KEL1*), *KEL2*) in the *C. neoformans* H99 strain background and found that Kem1 (Ral2) and Kem2 (Kel1), but not Kel2, are in part involved in mating. Otherwise we have not found any other phenotypes associated with these mutations. Capsule and melanin production seem to be normal in these strains, although more detailed analysis might be required. Currently, we are constructing *kem1 kem2* double mutant strains for further analysis.

Recent studies by other groups have implicated the kelch proteins Gpb1/2 in exerting an additional level of regulatory control, possibly via direct interactions with PKA. Our hypothesis is that the signaling components exist as components of a larger macromolecular complex, and this likely will provide insights into the functions of the human NF1 homolog. Our ongoing studies address the physical interaction binding partners for the protein kinase A catalytic subunits Tpk1 and Tpk2, and reveal that

protein kinase A is physically associated with RNA polymerase II in the nucleus. Taken together with recent studies from Rick Young's lab that provide evidence that PKA occupies chromatin at the promoters for regulatory target genes, these studies forge a link between signaling pathway components and direct nuclear control of gene expression.

Signaling pathways effectively modify expression of a large number of genes in response to nutritional stimuli to bring about dramatic changes in metabolic activity and protein synthesis capacity that are necessary for cellular adaptation to new environments. A good example is the rapid reprogramming of expression hundreds of genes in starved yeast cells upon encountering ample glucose, which activates the cAMP-dependent protein kinase A (PKA) pathway. How cells achieve such signaling efficacy remains poorly understood. Using affinity tagged protein subunits and mass spectrometry analyses, we have found that the catalytic subunits of yeast protein kinase A directly bind to RNA polymerase II upon activation. This physical interaction is mediated by the carboxy-terminal domain (CTD) of Rpb1, which is the largest subunit of the transcription machinery. Such physical coupling between active signaling molecules and transcription apparatus presumably provides a "molecular highway" that links an extra-cellular signal simultaneously to the transcription events at a large number of genomic loci. The role that the kelch proteins play in this novel aspect of PKA signaling is under current investigation and a manuscript describing these studies is in preparation to be submitted (see attached appendices).

In the remaining period of the approved no cost extension, we propose to complete aspects of the original aims of the proposal centered on the heterologous expression of NF1 in yeast cells, and to test the hypothesis that homologs of Gpb1 and Gpb2 kelch repeat proteins are expressed in mammalian cells that similarly govern the activity of neurofibromin.

Key Research accomplishments for years one, two, and three.

1. The kelch Gβ mimic Gpb1/2 proteins are recruited to the plasma membrane in a Gα Gpa2-dependent manner.
2. The kelch proteins Gpb1/2 were shown to function at the cell membrane.
3. The yeast neurofibromin homologs Ira1 and Ira2 were identified as physical and functional binding partners for the kelch proteins Gpb1/2.
4. Genetic and physical data support Ira1/2 as physiological targets of the kelch proteins Gpb1/2.
5. Regions of Ira1 and Ira2 that interact with Gpb1/2 were identified and found to be conserved in evolution.
6. Pulse-chase studies were conducted to establish that Gpb1/2 bind to and stabilize the yeast neurofibromin homologs Ira1/2 via the conserved interaction domain.
7. Homologs of the kelch proteins were identified in a divergent fungal species, enabling further molecular and genetic analysis of their roles in governing signaling via the cAMP pathway.
8. Mass spectrometric analysis was applied to identify and study proteins directly interacting with protein kinase A. A prominent interaction with RNA polymerase II was uncovered, providing key insights into how the kelch protein regulated protein kinase A pathway may participate in more direct control of transcription than previously appreciated.

Reportable outcomes

1. A senior research associate in my laboratory who is working on this project was awarded the Young Investigator Award from the Children's Tumor Foundation (formerly the National Neurofibromatosis Foundation) in 2004.
2. Our study entitled: "Gα subunit Gpa2 recruits kelch repeat subunits that inhibit receptor-G protein coupling during cAMP induced dimorphic transitions in *Saccharomyces cerevisiae*" was published in *Molecular Biology of the Cell* in 2005 (See Appendices).
3. Our study entitled: "The kelch proteins Gpb1 and Gpb2 inhibit Ras activity via association with the *Saccharomyces cerevisiae* RasGAP neurofibromin homologs Ira1 and Ira2" was published in *Molecular Cell* on June 23, 2006 (see appendices).
4. Our review on this topic entitled: "Sensing the environment: lessons from fungi" was published in Nature Reviews Microbiology in January 2007 (see appendices).
5. Toshiaki Harashima attended the international NF conference held near Park City, Utah June 10-12, 2007 and presented an invited talk entitled: "The kelch proteins Gpb1 and Gpb2 inhibit Ras activity via association with the yeast RasGAP neurofibromin homologs Ira1 and Ira2", see appendices for abstract and letter of invitation).

Conclusions

These studies have identified the Gpb1/2 binding domain (GBD) near the C-terminus of the neurofibromin homologs Ira1/2 and function to stabilize Ira1/2 enabling control of Ras signaling. Loss of Gpb1/2 results in a decrease in the RasGAP Ira1/2 proteins and consequently to an increase in the GTP bound form of Ras, which is the active form of Ras and ultimately associated with NF1. Importantly the GBD is significantly conserved in neurofibromin homologs, including the human counterpart, and mutations that lead to loss of the GBD have been identified from NF1 patients. Therefore the same regulatory mechanisms may be conserved in evolution, and this study should provide information as to how the RasGAP activity of neurofibromin is regulated and ultimately provide therapeutic clues for NF1 patients and possible avenues for novel drug development.

References

Ars, E., Kruyer, H., Morell, M., Pros, E., Serra, E., Ravella, A., Estivill, X., and Lázaro, C. (2003). Recurrent mutations in the *NF1* gene are common among neurofibromatosis type 1 patients. J Med Genet *40*, e82.

Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M., and Collins, F. (1990). The *NF1* locus encodes a protein functionally related to mammalian GAP and yeast *IRA* proteins. Cell *63*, 851-859.

Ballester, R., Michaeli, T., Ferguson, K., Xu, H. P., McCormick, F., and Wigler, M. (1989). Genetic analysis of mammalian GAP expressed in yeast. Cell *59*, 681-686.

Buchberg, A. M., Cleveland, L. S., Jenkins, N. A., and Copeland, N. G. (1990). Sequence homology shared by neurofibromatosis type-1 gene and *IRA-1* and *IRA-2* negative regulators of the *RAS* cyclic AMP pathway. Nature *347*, 291-294.

Dasgupta, B., and Gutmann, D. H. (2003). Neurofibromatosis 1: closing the GAP between mice and men. Curr Opin Genet Dev *13*, 20-27.

De Luca, A., Buccino, A., Gianni, D., Mangino, M., Giustini, S., Richetta, A., Divona, L., Calvieri, S., Mingarelli, R., and Dallapiccola, B. (2003). NF1 gene analysis based on DHPLC. Hum Mutat *21*, 171-172.

Fahsold, R., Hoffmeyer, S., Mischung, C., Gille, C., Ehlers, C., Kücükceylan, N., Abdel-Nour, M., Gewies, A., Peters, H., Kaufmann, D., *et al.* (2000). Minor lesion mutational spectrum of the entire *NF1* gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. Am J Hum Genet *66*, 790-818.

Harashima, T., and Heitman, J. (2002). The Gα protein Gpa2 controls yeast differentiation by interacting with kelch repeat proteins that mimic Gβ subunits. Mol Cell *10*, 163-173.

Harashima, T., and Heitman, J. (2005). Gα subunit Gpa2 recruits kelch repeat subunits that inhibit receptor-G protein coupling during cAMP induced dimorphic transitions in *Saccharomyces cerevisiae*. Mol Biol Cell *16*, 4557-4571.

Harashima, T., Anderson, S., Yates, J. R., and Heitman, J. The kelch proteins Gpb1 and Gpb2 inhibit Ras activity via assocation with the yeast RasGAP neurofibromin homologs Ira1 and Ira2, *Molecular Cell*, 22, 819-830, 2006.

Martin, G. A., Viskochil, D., Bollag, G., McCabe, P. C., Crosier, W. J., Haubruck, H., Conroy, L., Clark, R., O'Connell, P., Cawthon, R. M., and et al. (1990). The GAP-related domain of the neurofibromatosis type 1 gene product interacts with *ras* p21. Cell *63*, 843-849.

Origone, P., De Luca, A., Bellini, C., Buccino, A., Mingarelli, R., Costabel, S., La Rosa, C., Garrè, C., Coviello, D. A., Ajmar, F., *et al.* (2002). Ten novel mutations in the human neurofibromatosis type 1 (NF1) gene in Italian patients. Hum Mutat *20*, 74-75.

Parada, L. F. (2000). Neurofibromatosis type 1. Biochim Biophys Acta *1471*, M13-19.

Rasmussen, S. A., and Friedman, J. M. (2000). *NF1* gene and neurofibromatosis 1. Am J Epidemiol *151*, 33-40.

Tanaka, K., Lin, B. K., Wood, D. R., and Tamanoi, F. (1991). *IRA2*, an upstream negative regulator of RAS in yeast, is a RAS GTPase-activating protein. Proc Natl Acad Sci U S A *88*, 468-472.

Tanaka, K., Matsumoto, K., and Toh-e, A. (1989). *IRA1*, an inhibitory regulator of the *RAS*-cyclic AMP pathway in *Saccharomyces cerevisiae*. Mol Cell Biol *9*, 757-768.

Tanaka, K., Nakafuku, M., Satoh, T., Marshall, M. S., Gibbs, J. B., Matsumoto, K., Kaziro, Y., and Toh-e, A. (1990a). *S. cerevisiae* genes *IRA1* and *IRA2* encode proteins that may be functionally equivalent to mammalian *ras* GTPase activating protein. Cell *60*, 803-807.

Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K., and Toh-e, A. (1990b). *IRA2*, a second gene of *Saccharomyces cerevisiae* that encodes a protein with a domain homologous to mammalian *ras* GTPase-activating protein. Mol Cell Biol *10*, 4303-4313.

Upadhyaya, M., Osborn, M. J., Maynard, J., Kim, M. R., Tamanoi, F., and Cooper, D. N. (1997). Mutational and functional analysis of the neurofibromatosis type 1 (*NF1*) gene. Hum Genet *99*, 88-92.

Xu, G. F., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R., Weiss, R., and Tamanoi, F. (1990a). The catalytic domain of the neurofibromatosis type 1 gene product stimulates *ras* GTPase and complements *ira* mutants of *S. cerevisiae*. Cell *63*, 835-841.

Xu, G. F., O'Connell, P., Viskochil, D., Cawthon, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., White, R., and et al. (1990b). The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell *62*, 599-608.

Zhu, Y., and Parada, L. F. (2002). The molecular and genetic basis of neurological tumours. Nat Rev Cancer *2*, 616-626.

Appendices

Statement of Work

Task 1. To characterize the roles of the Gβ mimic kelch proteins Gpb1 and Gpb2 in regulating the yeast neurofibromin homologs Ira1 and Ira2 (Months 1-12):

a. Determine the role of Gpb1/2 on Ira1/2 (Months 1-4.5)
    I. Construct and develop materials required for GAP assay of Ira1/2 (Months 1-3).
    II. Perform GAP assay to examine the roles of Gpb1/2 on Ira1/2 RasGAP activity (Months 3-4.5).
b. Identify the Gpb1/2-binding domain on Ira1/2 (Months 1-6):
    I. Construct Ira1/2 derivatives carrying various deletions in the N-terminal, central, and C-terminal regions (Months 1-4.5).
    II. Test protein-protein interactions and identify the Gpb1/2-binding domain (Months 4.5-6).
c. Identify amino acid residues in Ira1/2 required for protein-protein interactions with Gpb1/2 (Months 6-12):
    I. Mutagenize the Gpb1/2-interacting domain in Ira1/2 and clone into the yeast two-hybrid vector (Months 6-7).
    II. Test protein-protein interactions and identify amino acids required for physical interactions with Gpb1/2 (Months 7-9).

III. Introduce mutations in the *IRA1/2* genes that abolish physical interactions with Gpb1/2 in vivo (Months 9-11).

IV. Test for pseudohyphal differentiation to characterize the role of the mutated amino acids in vivo (Months 11-12).

Task 2. To identify amino acid residues important for function of neurofibromin and Ira1/2 (Months 12-24):

a. Construct and express the *NF1* gene in yeast *ira1,2* mutants to examine whether the full length neurofibromin is functional when heterologously expressed in yeast cells (Months 12-13).

b. To identify putative Gpb1/2 binding sites in neurofibromin (Months 13-24):

   I. Introduce mutations in those ones of neurofibromin and clone these novel *NF1* alleles into yeast and mammalian expression vectors (Months 13-17).

   II. Express these *NF1* alleles in the yeast *ira1,2* mutant and mouse $NF1^{-/-}$ cells and characterize the roles of the mutated amino acids in vivo (Months 17-24).

c. To characterize the roles of the consensus PKA phosphorylation sites in neurofibromin and Ira1/2 (Months 13-24):

   I. Introduce mutations in candidate PKA phosphorylation sites in neurofibromin and Ira1/2 (Months 13-17).

II. Express these *NF1* mutant alleles in the yeast *ira1,2* mutant and mouse *NF1*$^{-/-}$ cells and the *IRA1/2* mutant alleles in the *ira1,2* mutant cells and test for phenotypes to examine the roles of those putative PKA phosphorylation sites (Months 17-24).

Task 3. To identify a human Gpb1/2 counterpart (Months 24-36):

a. To examine whether yeast Gpb1/2 interact with neurofibromin (Months 24-27):
    I. Construct FLAG-Gpb1/2 to be expressed and transfected into murine cells (Months 24-25).
    II. Examine protein-protein interactions by FLAG tag based immunopurification methods and western blots using anti-neurofibromin and anti-FLAG antibodies (Months 25-27).
b. To isolate a human Gpb1/2 counterpart (Months 27-36):
    I. Perform psi-BLAST searches against human sequence databases (Month 27).
    II. Make constructs for analysis in the yeast two-hybrid system and test protein-protein interactions between neurofibromin and putative Gpb1/2 counterparts (Months 27-31).

III. Also generate yeast two-hybrid constructs of the candidate Gpb1/2 binding domain in neurofibromin and screen human two-hybrid libraries to identify putative Gpb1/2 counterparts (Months 31-36).